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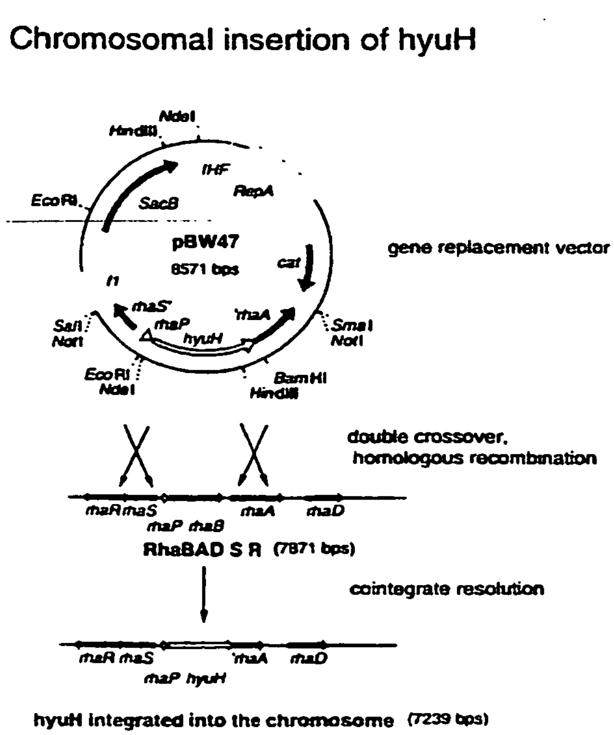
(54) Title: WHOLE CELL CATALYST COMPRISING A HYDANTOINASE, A RACEMASE AND A CARBAMOYLASE



(57) Abstract: A whole cell catalyst is described comprising a hydantoinase, a racemase and a carbamoylase. Thus this catalyst is able to degrade hydantoins directly into the amino acids. Additionally, a process for the production of this catalysts and for the production of amino acids is claimed.

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— *With (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description.*

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WHOLE CELL CATALYST COMPRISING A HYDANTOINASE, A RACEMASE AND A CARBAMOYLASE

The present invention is directed to a micro-organism, which is able to degrade hydantoins to enantiomerically enriched amino acids. Especially, this micro-organism is equipped with cloned genes coding for the necessary enzymes.

Racemic 5-monosubstituted hydantoins can be chemically synthesized according to Bucherer-Berg method using aldehydes, ammonium bicarbonate and sodium cyanide as reactants. They are important precursors for the enzymatic production of D-and L- amino acids. With the increasing demand for optically pure amino acids a lot of effort has been made towards the isolation of microorganisms capable for stereospecific hydrolysis of the hydantoins and characterization of the enzymes (Syldatk and Pietzsch, "Hydrolysis and formation of hydantoins" (1995), VCH Verlag, Weinhein, pp. 403-434; Ogawa et al., J. Mol. Catal. B: Enzym 2 (1997), 163-176; Syldatk et al., Appl. Microbiol. Biotechnol. 51 (1999), 293-309). The asymmetric bio-conversion to either L- or D- amino acids consists of three steps:

(i) chemical and/or enzymatic racemization of 5-substituted hydantoins

(ii) ring opening hydrolysis achieved by a hydantoinase and

(iii) hydrolysis of the N-carbamoyl amino acid produced by hydantoinase to the amino acid by carbamoylase.

Arthrobacter aurescens DSM 3747 is one of the few isolated microorganisms capable of converting 5-monosubstituted hydantoins to L-amino acids. The disadvantage of using A. aurescens cells as biocatalyst is the low enzyme activity. Especially the L-N-carbamoylase is the bottleneck for most

substrates leading to an increase of the intermediate L-N-carbamoyl amino acid in the cell, which is not further converted to the corresponding amino acid. By combining the purified enzymes bottlenecks could be avoided but due to the low amounts of enzymes in the cells and loss of activity during the many necessary purification steps this process is not cost-effective.

All three genes encoding for the racemase hyuA (seq. 11), the L-specific hydantoinase hyuH (seq. 9) and the stereoselective L-N-carbamoylase (seq. 7) have been cloned in E. coli separately and expressed to high levels (about 10 % of the total cell protein)(DE 19913741; J. Biotechnol., to be published). For in vitro catalysis the enzymes from the three recombinant strains can be produced and purified more cost-effective then from the Arthrobacter aurescens strain. Regarding the different enzyme activities towards the various substrates the enzymes can be combined in enzyme reactors at ratios optimized for each reaction.

It is an object of this invention to provide a further possibility of how a racemase, a hydantoinase and a D- or L-specific carbamoylase can act together in a process for the production of enantiomerically enriched amino acids from 5-monosubstituted hydantoins. Especially, this possibility should be suitable to be implemented in processes on technical scale, that is to say it has to be most cost-effective.

This is done by using a whole cell catalyst according to claim 1. Further preferred catalysts are subjects to claims depending from claim 1. Claims 6 to 9 are directed to a process for the production of the whole cell catalyst of the invention. Claims 10 and 11 protect a process for the production of enantiomerically enriched amino acids using the catalyst according to the invention.

Using whole cell catalysts comprising cloned genes encoding for a hydantoinase, for a hydantoin racemase and a D- or L-specific carbamoylase for the conversion of 5-monosubstituted hydantoins to L- or D-amino acids results in a fast and complete conversion of racemic mixtures of hydantoins to the corresponding L- or D-amino acids on industrial scale. This significantly reduces the production costs due to a reduction of fermentation and purification costs because all enzymes are produced in one strain.

Advantageously, a bacteria is used as cell, because of high reproduction rates and easy growing conditions to be applied. There are several bacteria known to the skilled worker which can be utilized in this respect. Preferably a Escheria coli can be used as cell and expression system in this regard (Yanisch-Perron et al. Gene (1985), 33, 103-109).

It is another positive embodiment of this invention that in principle all genes encoding for the hydantoinase, racemase and carbamoylase known to the artisan can be taken to be expressed in the whole cell catalyst. Preferably all genes can be taken from DSM 3747 (seq. 7, 9, 11).

The enzymes to be incorporated in the genetic code of the whole cell catalyst naturally possess different turnover rates. It is a drawback if the rates of co-working enzymes are not in line and intermediates accumulate during the production inside the cell. The overexpression of the hydantoinase gene in E. coli leads to the formation of inclusion bodies (Wiese et al., in preparation), which is unfavourable for a well balanced coexpression of all the three enzymes. Therefore, various attempts to "fine tune" the expression of these genes have been made. This can be done advantageously by overexpressing the hydantoinase genes in question according to their turnover rates. According to the DSM 3747-System the hydantoinase gene is overexpresses from plasmids with reduced copy numbers.

A further embodiment of the instant invention is directed to a process for the production of the whole cell catalyst according to the invention. In principle all plasmids known to the skilled worker can serve to carry the gene into the expression system. Preferably, plasmids derived from pSC101, pACYC184 or pBR322 are used to produce the catalyst. Most preferably plasmids pBW31 and pBW32, pBW34 and pBW35, pBW34 and pBW53, pBW32 or pBW34 are used in this respect. For the skilled worker plasmids and methods to produce plasmids can be deduced from Studier et al., Methods Enzymol. 1990, 185, 61-69 or brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. More applicable plasmids, vectors can be found in:
DNA cloning: a practical approach. Volume I-III, edited by D. M. Glover, IRL Press Ltd., Oxford, Washington DC, 1985, 1987; Denhardt, D. T. and Colasanti, J.: A surey of vectors for regulating expression of cloned DNA in E. coli. In: Rodriguez, R.L. and Denhardt, D. T (eds), Vectors, Butterworth, Stoneham, MA, 1987, pp179-204;
Gene expression technology. In: Goeddel, D. V. (eds), Methods in Enzymology, Volume 185, Academic Press, Inc., San Diego, 1990; Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2^{nd} ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. They are incorporated by reference herewith.

Over-expression can be accomplished be means known to the skilled artisan, e.g. using constitutive or inducible expression systems as reviewed by Makrides (Makrides, 1996, Microbiol. Rev. 60, no. 3, 512-538)

Preferably, for expression of the enzymes a rhamnose inducible E. coli promoter cassette is used.

In addition, primers useful for the amplification of the gene of the invention in a PCR are protected similarly. Primers which are feasible are for example, primers S988 (seq. 6), S2480 (seq. 1), S2248 (seq. 2), S2249(seq. 3),

S2517 (seq. 4) or S2518 (seq. 5). Furthermore, all other primers which could serve to carry out this invention and which are known to the artisan are deemed to be useful in this sense. The finding of a suitable primer is done by comparison of known DNA-sequences or translation of amino acid sequences into the codon of the organism in question (e.g. for Streptomyces: Wright et al., Gene 1992, 113, 55-65). Similarities in amino acid sequences of proteins of so called superfamilies are useful in this regard, too (Firestine et al., Chemistry & Biology 1996, 3, 779-783). Additional information can be found in Oligonucleotide synthesis: a practical approach, edited by M.J. Gait, IRL Press Ltd, Oxford Washington DC, 1984; PCR Protocols: A guide to methods and applications, edited by M.A. Innis, D.H. Gelfound, J.J. Sninsky and T.J. White. Academic Press, Inc., San Diego, 1990. Those strategies are incorporated by reference herewith.

Another aspect of the invention is a process for the production of enantiomerically enriched amino acids, which utilizes a whole cell catalyst according to the invention. Furthermore, a process is preferred that is performed in an enzyme-membrane-reactor (DE 19910691.6).

To adopt the turnover rate of all enzymes expressed in the whole cell catalyst to each other there are different methods to achieve this.

a) The genes are expressed with different promoters. The gene with the lowest activity is combined with the strongest promoter and vice versa. A disadvantage would be that for each gene a different inductor is necessary to induce the expression of all genes.

b) The genes are expressed with one promoter on a polycistronic messenger. The ratios of synthesis of the enzymes is influenced by changing or by mutation of the translation initiation region of each gene (the ribosomal

binding site) which determines the efficiency of protein synthesis. This principle is realized in operons of microorganisms. The disadvantage is that the efficiency of a translation initiation region can not be predicted which means that for each gene many changes in the translation initiation region have to be made and tested (Grifantini et al., 1998, Microbiology, 144, 947-954).

c) The enzyme activity of each enzyme can be changed by mutation using error prone PCR (Fromant et al., 1995, Anal. Biochem. 224, 347-353) and DNA shuffling (Stemmer, 1994, Nature 370, 389-391). Again, this is very time consuming and costly.

d) Instead of mutagenizing genes to optimize their function in a reaction cascade, genes from different origins which encode enzymes with appropriate properties could be combined to an operon. This needs a large database describing such enzymes.

e) All genes are expressed from the same promoter but from replicons with different copy numbers. This can be the chromosome (single copy) or plasmids with low, moderate and high copy numbers. By constructing various compatible plasmids with different copy numbers and antibiotic markers carrying each the same cassette with the promoter and a polylinker sequence, genes of interest can be integrated into the plasmids in one step and the plasmids combined within one strain. This method allows a fast construction and testing of many combinations and with just one inducer in one fermentation all genes are expressed at different levels according to the plasmid copy number.

The following paragraphs show the transformation of Hydantoins to enantiomerically enriched amino acids.

Expression of the hyuA gene (seq. 11)is necessary for complete substrate conversion. Figure 1 shows the time

course of conversions with E.coli BW3110 containing pAW229 and pBW31. pAW 229 contains the carbamoylase gene on a pACYC plasmid, pBW31 is a pBR derivative and carries the hydantoinase gene. After consumption of 50% of the substrate, the reaction almost stops completely, since spontanous racemisation of IMH is very slow (Syldatk et. al., "Biocatalytic production of amino acids and derivatives" (1992), Hanser publishers, New York, pp. 75-176). As can be seen from figure 2, bringing the racemase (seq. 11) into the system by using pBW31 and pBW32, the pACYC plasmid with the carbamoylase and the racemase gene, enables complete conversion of the substrate. After 4.5 hours induction at 30°C 200 µl permeabized cells were prepared as described above and were incubated with 800 µl of 2 mM D,L-IMH.
The E. coli strain BW3110H with the chromosomally integrated hydantoinase gene was transformed with pBW32 (Figure 3), the pACYC plasmid containing the carbamoylase and the racemase gene, or with pBW34 (Figure 4), the pBR plasmid containing the carbamoylase and the racemase gene. Cells were induced at 25°C for 8.5 hours (pBW32), or for 11.5 hours (pBW34). Cell harvesting and permeabilization took place as described above.

Figure 5 shows E. coli BW3110 cells with pBW31 (the pBR plasmid which carries the hydantoinase gene) and pBW32 (the pACYC plasmid with the carbamoylase and racemase genes). Cells were induced 10 hours at 30°C.

The combination of pBW31 and 32 enables fast and complete conversion from D-L-IMH to tryptophane. The intermediate is formed up to a concentration of 0.4 mM.

E. coli BW3110 transformed with the plasmids pBW34 (the pBR plasmid with the carbamoylase and the racemase genes) and pBW35 (the pACYC plasmid with the hydantoinase gene) was taken for this conversion. Cells were induced for 10 hours at 30°C (Fig. 6). The combination of pBW34 and 35 shows an

accumulation of the intermediate up to over 1mM. The product formation takes place at a lower rate than seen in figure 5.

Plasmid pBW34 (the pBR plasmid with the carbamoylase and the racemase genes) was combined with pBW53 (the pSC101 plasmid with the hydantoinase gene). Induction took place for 10 hours at 30°C (Fig 7). In this case a fast conversion of IMH to tryptophane takes place. Formation of the intermediate is strongly reduced and product formation is faster than seen in figure 5, so that the combination of pBW34 and pBW53 is most favourable for the process.

The present invention shows a new and superior way to combine a hydantoinase, a hydantoin racemase and a carbamoylase in a whole cell catalysator. It is this possibilty that renders instant invention to a proper method for the production of enantiomerically enriched amino acids from hydantoins due to reduction of catalyst production costs.

Enantiomerically enriched means that one antipode of a chiral compound is the major component in a mixture of both antipodes.

Amino acid denotes within the framework of this invention all compounds comprising a primary amine function connected to a carboxylic acid group via one intermediate C-atom (α-C-atom). This α-C-atom bears only one further residue. Nevertheless all natural and unnatural amino acids are deemed to be encompassed. Preferred unnatural amino acids are those mentioned in DE 19903268.8.

Genes encoding for a peptide sequence are to be understood as all genes possible with regard to the degeneration of the genetic code.

The microorganism DSM 3747 is disposited at Deutsche Sammlung für Mikroorganismen und Zellkulturen.

Examples:

Bacterial strains, plasmids and growth conditions:
E. coli JM109 (Yanisch-Perron et al. Gene (1985), 33, 103-109) was used for cloning procedures involving the hyuC (seq. 7), hyuH (seq. 9) and hyuA (seq. 11) genes from Arthrobacter aurescens DSM 3747 (Groß et al., Biotech. Tech. (1987), 2, 85-90). E. coli BW3110 (Wilms et al, in preparation), a derivative of E. coli W3110 (Hill and Harnish, 1981 Proc. Natl. Acad. Sci USA 78, 7069-7072) was used for coexpression for the genes mentioned above. E. coli strains were either grown in LB liquid medium or on LB-agar plates (Luria et al., 1960, Virology 12, 348-390), both supplemented with 100 µg/ml ampicillin and / or 25µg/ml chloramphenicol to select plasmid carrying strains. The cultures were grown at 37°C, for heterologous gene expression growth temperature was reduced to 30°C or 25°C.

General protocols:
All of the recombinant DNA techniques were standard methods (Sambrook et al., Molecular Cloning: A laboratory manual (1989), Cold Spring Habour Laboratory Press, New York). PCR reactions were performed either with Pwo Polymerase or the Expand™ Long Template PCR System by following the recommendations of Roche Diagnostics.

Coexpression of hyuA, hyuC, and hyuH in E. coli:
For coexpression of the racemase gene hyuA, the carbamoylase gene hyuC, and the hydantoinase gene hyuH in E. coli, several constructions with different features were made. To obtain comparable expression levels of the genes, variations in the copy number of plasmids were used. High copy plasmids like pBR plasmids (Bolivar et al., 1977, Gene 22, 277-288) have a copy number of 40-50. PACYC184 plasmids (Chang and Cohen, 1978, J. Bacteriol.,1141-1156) have a copy number of 10-15. PSC101 plasmids (Cohen et al., 1973, Proc. Natl. Acad. Sci. USA, 70, 3240-3244) have a copy

number of 5-10. A copy number of 1 is achieved by inserting the gene into the E. coli chromosome.

The plasmid features are summarized in table1:

plasmid name	ori	copy number	resistance	hyu - genes
pAW229	pACYC	10-15	cam	hyuC
pBW31	pBR	40-50	amp	hyuH
pBW32	pACYC	10-15	cam	hyuC + hyuA
pBW34	pBR	40-50	amp	hyuC + hyuA
pBW35	pACYC	10-15	cam	hyuH
pBW53	pSC101	5-10	cam	hyuH

Abbreviations: hyu: hydantoin utilizing

hyuA: racemase gene (seq. 11)

hyuC: carbamoylase gene (seq. 7)

hyuH: hydantoinase gene (seq. 9)

amp: ampicillin resistance (ß-lactamase gene)

cam: chloramphenicol resistance (chloramphenicol acetyl transferase gene)

The hydantoinase gene hyuH was also expressed using the strain BW3110H, which carries a chromosomal insertion of the hyuH gene.

All constructs enable transcriptional regulation of gene expression by the rhaBAD promoter.
For coexpression of the carbamoylase gene hyuC and the hydantoinase gene hyuH pAW229 and pBW31 are transformed into E. coli BW3110.
For coexpression of the racemase gene hyuA, the carbamoylase gene hyuC and the hydantoinase gene hyuH, pBW31 and pBW32, pBW34 and pBW35, or pBW34 and pBW53 are suitable combinations in E. coli BW3110.
To achieve coexpression of all three Enzymes in E. coli BW3110H, pBW32 or pBW34 can be used.

Construction of the plasmids:
pAW229 was obtained by cleaving pAW178 (Wilms et al., J. Biotechnol. (1999), 68, 101-113) with the restriction enzymes NdeI and BamHI and ligating the 1241bp fragment containing the hyuC gene into pJOE2962 (Altenbuchner, unpublished), which was cut with the same restriction enzymes.
pBW31 was constructed by cleaving pAW92 (Wiese et al., in preparation) with the restriction enzymes EcoRI and BamHI and ligating the 1436bp fragment containing the hyuH gene into pBW22, which was cut with the same restriction enzymes.
pBW32 was obtained by PCR amplification of the hyuA gene using the primers S988 (5´-AGGCTGAAAATCTTCTCT-3`) (seq. 6) and S2480 (5´-AAAAAAGCTTTTAAGAAGGAGATATACATA-3`) (seq. 1) and pAW210 (Wiese et al., in preparation) as template. Included in primer S2480 is a shine dalgarno sequence for translation initiation. The fragment was inserted into the HindIII site of pAW229.
pBW34 was created by inserting the hyuA PCR fragment described above into the HindIII site of pBW24. pBW24 was

obtained by cleaving pAW178 (Wilms et al, J. Biotechnol. (1999), 68, 101-113) with NdeI and HindIII and ligating the 1261bp long fragment containing the hyuC gene into pBW22, which was cut with the same restriction enzymes. pBW22 was constructed by PCR amplifying of the "cer"-region from the colE1 plasmid using the primers S2248 (5´-AAA GCA TGC ATG GCC CTT CGC TGG GAT-3´) (seq. 2) and S2249 (5´-AAA GCA TGC ATG GCT ACG AGG GCA-3´) (seq. 3). The 268bp fragment was cut with the restriction enzyme SphI and inserted in the vector pJOE2775 (Krebsfänger et al., 1998, Enzyme Microb. Technol. 22, 219-224) which was cut with the same restriction enzyme.

pBW35 was constructed by cleaving pBW31 with the restriction enzymes NdeI and BamHI. The 1379bp fragment containing hyuH was inserted into pAW229, which was cut with the same restriction enzymes.

pBW53 was obtained by cleaving pBW31 with the restriction enzymes SphI and BamHI. The 1534bp fragment containing the hyuH gene and the rhamnose promoter was inserted into pSB27 (Baumann, Dissertation, Universität Stuttgart, 1996), which was cut with the same restriction enzymes.

Construction of the chromosomal integrate of hyuH into the rhamnose operon:

A 3.5kb fragment from the E. coli rhamnose operon was amplified using the primers S2517 (5´-AAACAAGATCTCGCGACTGG-3´) (seq. 4) and S2518 (5´-AAAAAGATCTTTATCAGGCCTACAACTGTTG-3´) (seq. 5) and E. coli chromosomal DNA as template. The fragment was cut with the restriction enzyme BglII and inserted into the vector pIC20H (Marsh et al., 1984, Gene 32, 481-485), which was cut with the restriction enzymes BamHI and BglII, to get pBW39. PBW31 was cut with the restriction enzymes EcoRI and BamHI. The 1436bp fragment containing the hyuH gene was inserted into the vector pBW39, which was also cut with the same restriction enzymes, to get pBW40. A 2.9kb fragment was amplified using the primers S2517 and S2518 and pBW40 as a template. This

fragment was cut with BglII and inserted into the vector pJOE2114 (Altenbuchner, unpublished) which was also cut with BglII to get pBW45. PBW45 was cut with BglII and SphI. The resulting 2.9kb rhaS-rhaP-hyuH-rhaA fragment was inserted into the gene replacement vector pKO3 (Link et al, 1997, J. Bacteriol., 179, 20, 6228-6237), which was cut with BamHI. The gene replacement was carried out according to the authors´ instructions. Positiv insertion events were screened using MacConckey Rhamnose plates.

Preparation of cells and activity measurements:
For induction of the *rhaBAD* promoter strains with two plasmids were grown in $LB_{amp+cam}$, strains with one plasmid in LB_{amp} or LB_{cam} respectively to OD_{600} = 0.3-0.5. Then L-rhamnose was added to a final concentration of 0.1 g l^{-1} and the cultivation was continued to a final OD of approximately 5. If not indicated seperately, for small scale enzyme measurements cells corresponding to OD_{600} of 20 were harvested, washed in 1 ml 0.2 M Tris pH 7.0 and resuspended in 1 ml 0.2 M Tris pH 7.0, 1 mM $MnCl_2$. 10 µl toluene was added for permeabilizing the cell membranes. After 30min of incubation at 37°C 200 µl of this cell suspension were added to 800 µl of 2 mM D,L-Indolylmethylhydantoin (IMH) in 0.1 M Tris pH 8.5, mixed and shaked at 37°C. This cell amount corresponds to approximately 5-6 mg cell wet weight. Samples were taken regularly. The reaction was stopped by adding 14% trichloracetic acid. The time course of product and educt concentrations was determined using HPLC analysis. The HPLC-system was equipped with a RP-18 column as described previously for the determination of hydantoin derivatives and N-carbamoyl amino acids (May et al., 1998, J. Biotechnol., 26, 61 (1): 1-13). UV-absorption was measured at 280 nm and the mobile phase (0.3% (v/v) phosphoric acid (80%) and methanol (20%; v/v)) was pumped with a flow rate of 1.0 ml min^{-1}.

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0-1	Form - PCT/RO/134 (EASY) Indications R lating to Deposited Microorganism(s) r Oth r Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.91 (updated 01.07.2000)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	990160 AM

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	8
1-2	line	32
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	
1-3-4	Accession Number	DSMZ 3747
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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Claims:

1. Whole cell catalyst for the conversion of 5-monosubstituted hydantoins to L- or D-amino acids comprising cloned genes encoding for a hydantoinase, for a hydantoin racemase and a D- or L-specific carbamoylase.

2. Catalyst according to claim 1, characterized in that a bacteria is used as cell.

3. Catalyst according to claim 1 and 2, characterized in that Escherichia coli is used as cell.

4. Catalyst according to one or more of preceding claims, characterized in that the genes encoding for the hydantoinase, racemase and carbamoylase is taken from DSM 3747.

5. Catalyst according to one or more of preceding claims, characterized in that the genes are overexpressed in the cell according to their turnover rates.

6. Process for the production of a whole cell catalyst, characterized in that plasmids derived from pSC101, pACYC184 or pBR322 are used.

7. Process according to claim 6, characterized in that plasmids pBW31 and pBW32, pBW34 and pBW35, pBW34 and pBW53, pBW32 or pBW34 are used.

8. Process according to claim 6, characterized in that an E. coli strain with a chromosomally insertion of the hydantoinase gene is used.

9. Process according to claim 6, characterized in that a rhamnose inducible E. coli promoter cassette is used.

10. Process according to claim 6, characterized in that primers S2480, S2248, S2249, S2517 or S2518 are used.

11. Process for the production of enantiomerically enriched amino acids, characterized in that a whole cell catalyst according to claim 1 is used.

12. Process according to claim 11, characterized in that the process is performed in an enzyme-membrane-reactor.

13. Process for the production of whole cell catalysts characterized in that all genes are expressed from the same promotor but from plasmids with replicons with different copy numbers.

14. Plasmids pBW31, pBW32, pBW34, pBW35, pBW53, AW229.

15. Primers S2480, S2248, S2249, S2517, S2518.

16. Microorganisms comprising plasmids according to claim 14.

AMENDED CLAIMS

[received by the International Bureau on 6 February 2001 (06.02.01); original claims 1-16 replaced by new claims 1-15 (2 pages)]

1. Whole cell catalyst for the conversion of 5-monosubstituted hydantoins to L- or D-amino acids comprising cloned genes encoding for a hydantoinase, for a hydantoin racemase and a D- or L-specific carbamoylase, wherein the genes are overexpressed in the cell according to their turnover rates.

2. Catalyst according to claim 1, characterized in that a bacteria is used as cell.

3. Catalyst according to claim 1 and 2, characterized in that Escherichia coli is used as cell.

4. Catalyst according to one or more of preceding claims, characterized in that the genes encoding for the hydantoinase, racemase and carbamoylase is taken from DSM 3747.

5. Process for the production of a whole cell catalyst according to claim 1, characterized in that plasmids derived from pSC101, pACYC184 or pBR322 are used.

6. Process according to claim 5, characterized in that plasmids pBW31 and pBW32, pBW34 and pBW35, pBW34 and pBW53, pBW32 or pBW34 are used.

7. Process according to claim 5, characterized in that an E. coli strain with a chromosomally insertion of the hydantoinase gene is used.

8. Process according to claim 5, characterized in that a rhamnose inducible E. coli promoter cassette is used.

9. Process according to claim 5, characterized in that primers S2480, S2248, S2249, S2517 or S2518 are used.

10. Process for the production of enantiomerically enriched amino acids, characterized in that a whole cell catalyst according to claim 1 is used.

11. Process according to claim 10, characterized in that the process is performed in an enzyme-membrane-reactor.

12. Process for the production of whole cell catalysts according to claim 1 characterized in that all genes are expressed from the same promotor but from plasmids with replicons with different copy numbers.

13. Plasmids pBW31, pBW32, pBW34, pBW35, pBW53, AW229.

14. Primers S2480, S2248, S2249, S2517, S2518.

15. Microorganisms comprising plasmids according to claim 13.

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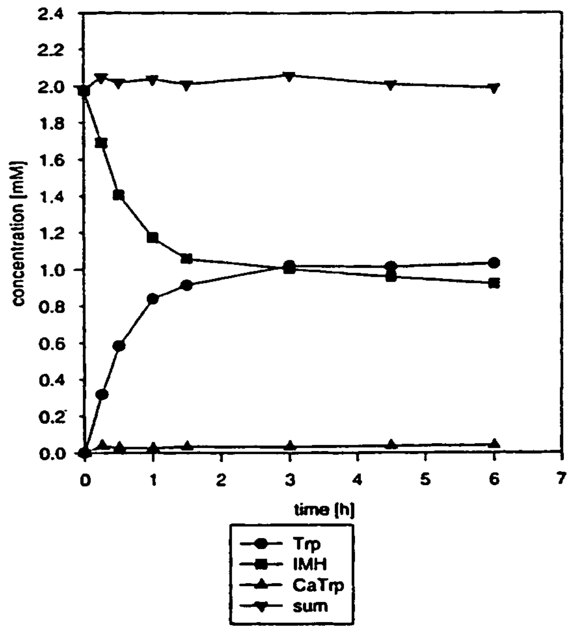
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sum

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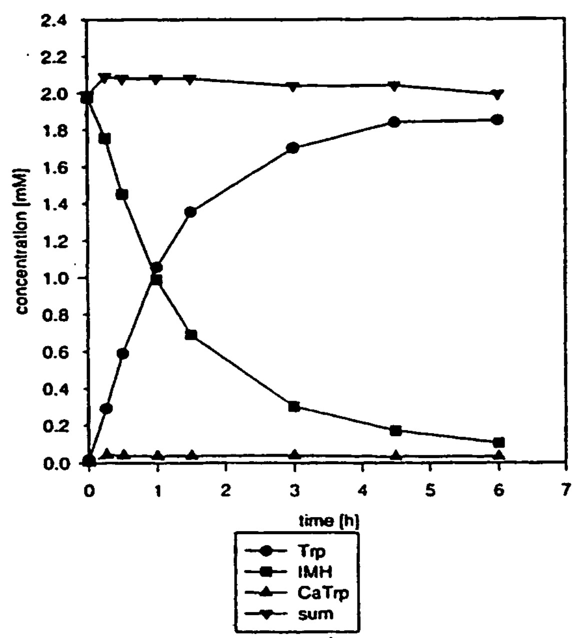
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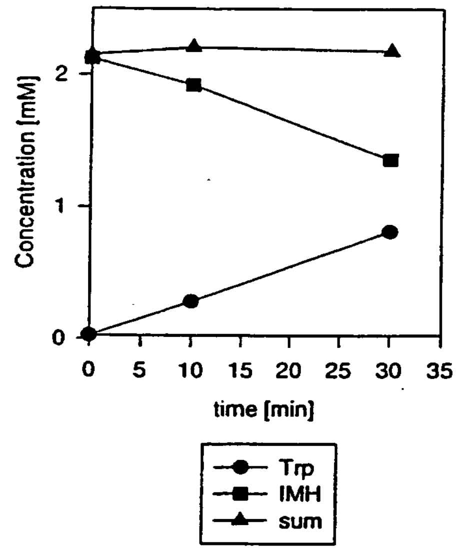
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IMH
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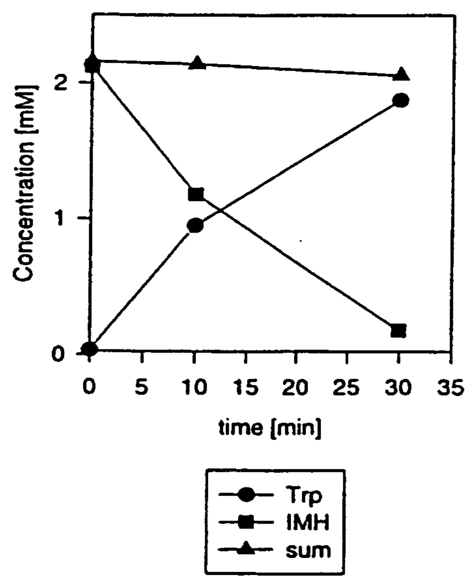
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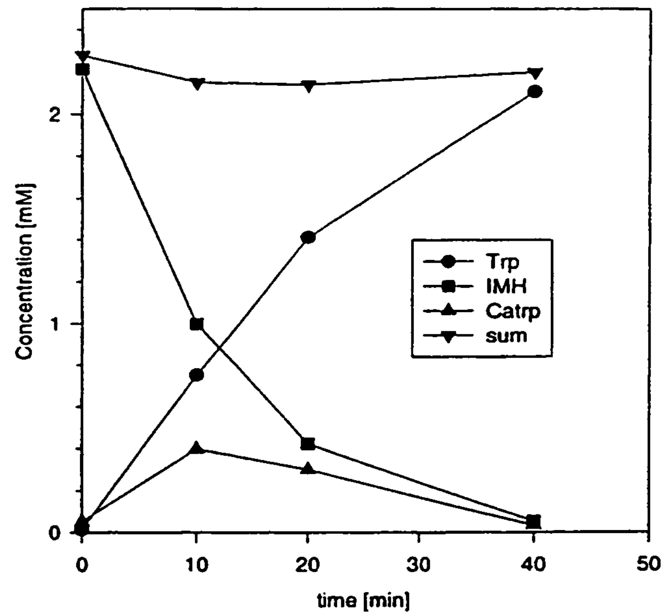
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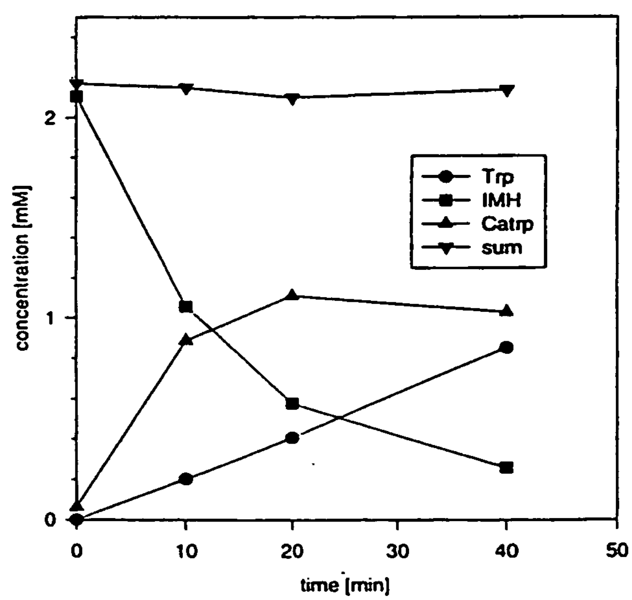
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50

4/10

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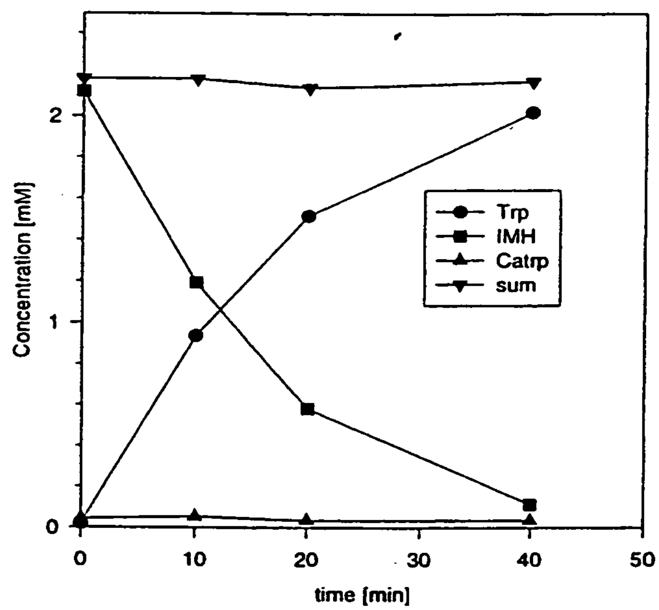
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IMH
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Construction of pAW229

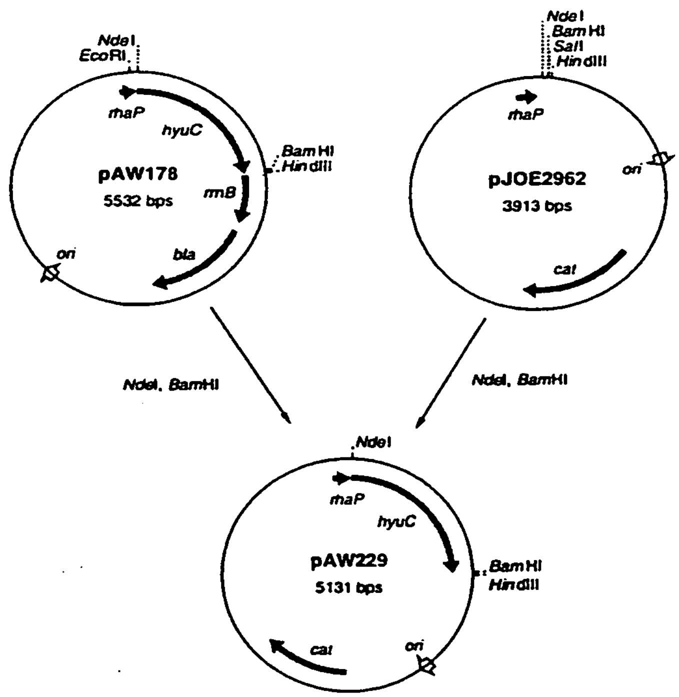


Fig. 9:

Construction of pBW31

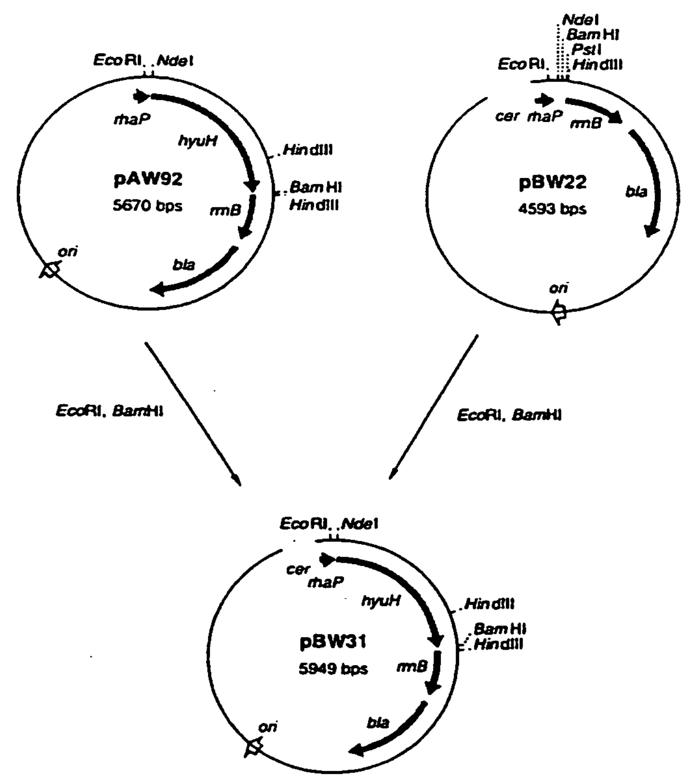


Fig. 10:

Construction of pBW32

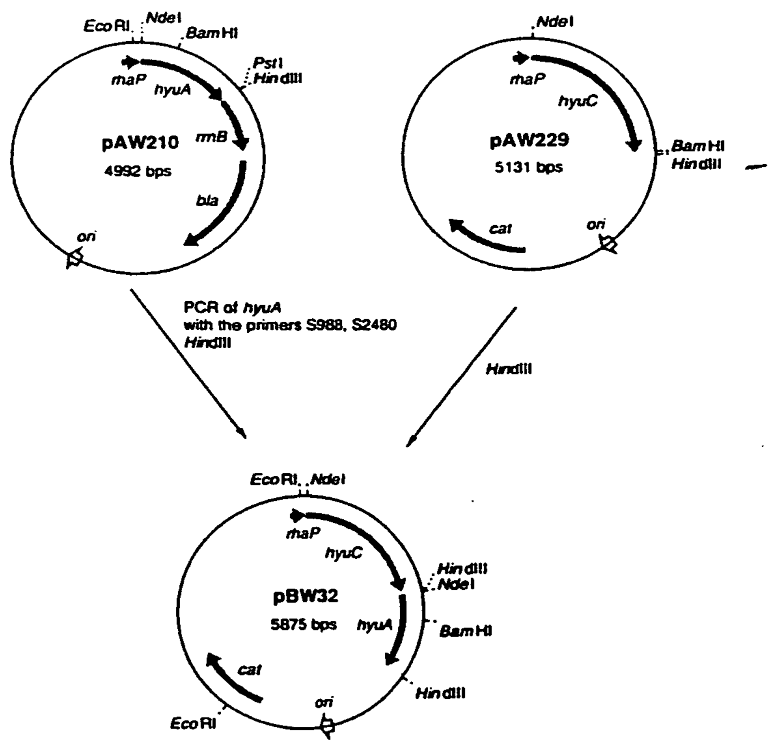


Fig. 11:

Construction of pBW34

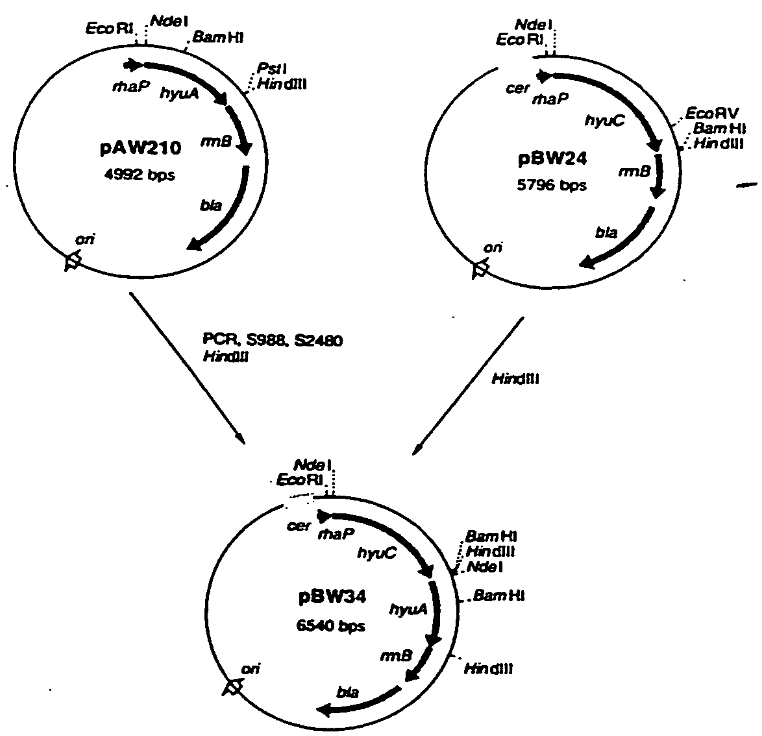


Fig. 12:

Construction of pBW53

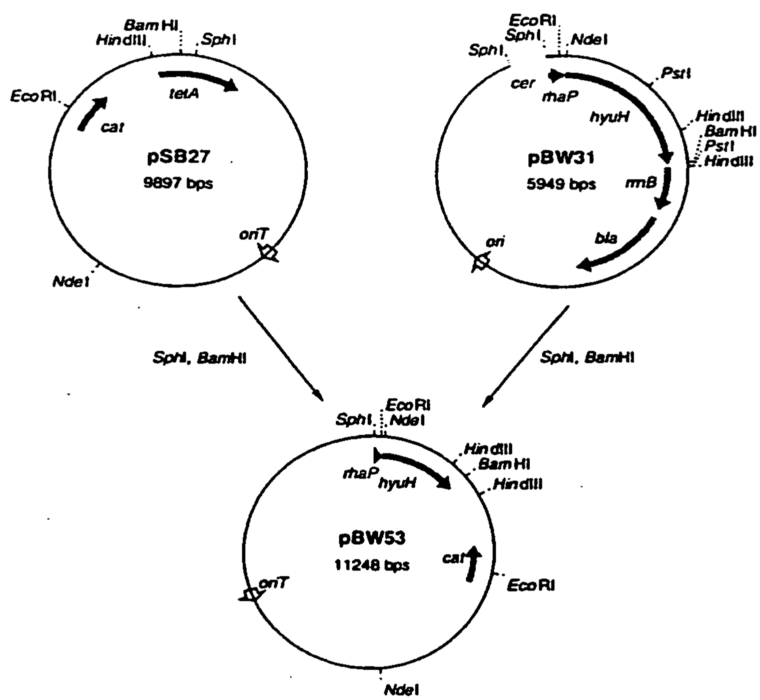
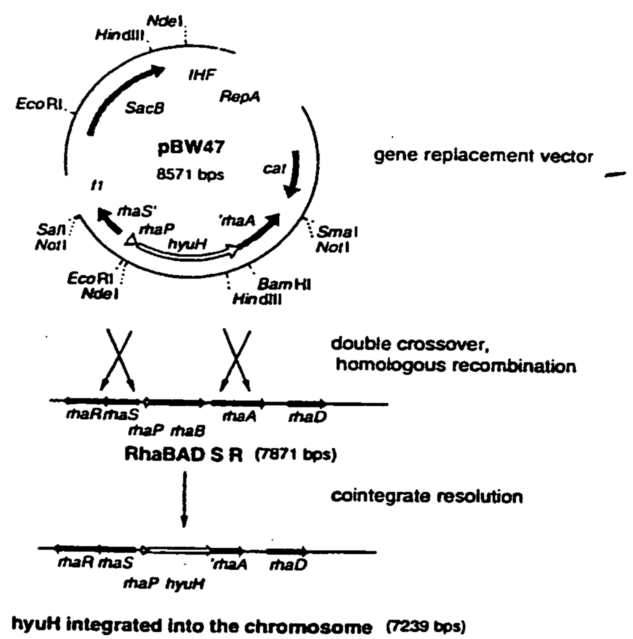


Fig. 13:

Chromosomal insertion of hyuH



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His Ile Glu Gln Gly Pro Ile Leu Glu Gln Glu Gln Ile Glu Ile Gly
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145                 150                 155                 160

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Pro Gly Leu Asp Ala Val Lys Glu Leu Thr Asp Arg Pro Val Val Gly

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gtt gcc gaa gct gca atc cac atg tct tca ttc gtc gcg gcc acc ttc    336
Val Ala Glu Ala Ala Ile His Met Ser Ser Phe Val Ala Ala Thr Phe
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tcc att gtc agc atc ctc ccg agg gtc agg aaa cat ctg cac gaa ctg    384
Ser Ile Val Ser Ile Leu Pro Arg Val Arg Lys His Leu His Glu Leu
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Val Arg Gln Ala Gly Ala Thr Asn Arg Leu Ala Ser Ile Lys Leu Pro
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Asn Leu Gly Val Met Ala Phe His Glu Asp Glu His Ala Ala Leu Glu
145                 150                 155                 160

acg ctc aaa caa gcc gcc aag gag gcg gtc cag gag gac ggc gcc gag    528
Thr Leu Lys Gln Ala Ala Lys Glu Ala Val Gln Glu Asp Gly Ala Glu
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tcg ata gtg ctc gga tgc gcc ggc atg gtg ggg ttt gcg cgt caa ctg    576
Ser Ile Val Leu Gly Cys Ala Gly Met Val Gly Phe Ala Arg Gln Leu
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        195                 200                 205

cgc gtg gcc gag agt ttg gtc gct ctg ggc tac cag acc agc aaa gcg    672
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115 120 125

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Ser Asp Glu Leu Gly Val Pro Val Ile Asp Pro Val Glu Ala Ala Cys
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Asn Ser Tyr Gln Lys Pro Thr Glu Lys Gln Tyr Leu
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370 375 380

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Ser His Phe Asp Ser Val Arg Asn Gly Gly Met Phe Asp Gly Thr Ala
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Gln Ala Ala Ala Arg Ser Ala Ala Asp Leu Arg Ala Phe Ile Glu Leu
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Ala Pro Gly Gly Gly Asn Gln Val Pro Gly Glu Val Asp Phe Thr Leu
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Asp Leu Arg Ser Pro His Glu Glu Ser Leu Arg Val Leu Ile Asp Arg
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65 70 75 80

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ttc cca ccc acc acc act ctg gac gcc ttc ctt gaa aag aag aag cag 336
Phe Pro Pro Thr Thr Thr Leu Asp Ala Phe Leu Glu Lys Lys Lys Gln

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                100                     105                     110

gcg ggg cag cgg ttg aaa gtt gac ttc gcg ctc tat gga ggt gga gtg     384
Ala Gly Gln Arg Leu Lys Val Asp Phe Ala Leu Tyr Gly Gly Gly Val
        115                     120                     125

ccg gga aac ctg ccc gag atc cgc aaa atg cac gac gcc ggc gct gtg     432
Pro Gly Asn Leu Pro Glu Ile Arg Lys Met His Asp Ala Gly Ala Val
    130                     135                     140

ggc ttc aag tca atg atg gca gcc tca gtg ccg ggc atg ttc gac gcc     480
Gly Phe Lys Ser Met Met Ala Ala Ser Val Pro Gly Met Phe Asp Ala
145                     150                     155                     160

gtc agc gac ggc gaa ctg ttc gaa atc ttc caa gag atc gca gcc tgt     528
Val Ser Asp Gly Glu Leu Phe Glu Ile Phe Gln Glu Ile Ala Ala Cys
                165                     170                     175

ggt tca gtc atc gtg gtt cat gcc gag aat gaa acg atc att caa gcg     576
Gly Ser Val Ile Val Val His Ala Glu Asn Glu Thr Ile Ile Gln Ala
            180                     185                     190

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gag gca tcc caa cca gtt ttc cag gag aac gag gcc att cag cgt gcg     672
Glu Ala Ser Gln Pro Val Phe Gln Glu Asn Glu Ala Ile Gln Arg Ala
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            260                     265                     270

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305                     310                     315                     320

aaa gaa ccc ggc tgg aag gac gtg tgg aaa gcc ggc aac ggt gcg ctg    1008
Lys Glu Pro Gly Trp Lys Asp Val Trp Lys Ala Gly Asn Gly Ala Leu
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370 375 380

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Gly Lys Phe Val Met Pro Gly Val Val Asp Glu His Val His Ile Ile
50 55 60

Asp Met Asp Leu Lys Asn Arg Tyr Gly Arg Phe Glu Leu Asp Ser Glu
65 70 75 80

Ser Ala Ala Val Gly Gly Ile Thr Thr Ile Ile Glu Met Pro Ile Thr
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Phe Pro Pro Thr Thr Thr Leu Asp Ala Phe Leu Glu Lys Lys Lys Gln
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16

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Pro Gly Asn Leu Pro Glu Ile Arg Lys Met His Asp Ala Gly Ala Val
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Gly Phe Lys Ser Met Met Ala Ala Ser Val Pro Gly Met Phe Asp Ala
145                 150                 155                 160

Val Ser Asp Gly Glu Leu Phe Glu Ile Phe Gln Glu Ile Ala Ala Cys
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225                 230                 235                 240

Ser Asn Pro Asp Gly Val Glu Leu Ile His Gln Ala Gln Ser Glu Gly
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Gln Asp Val His Cys Glu Ser Gly Pro Gln Tyr Leu Asn Ile Thr Thr
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Asp Asp Ala Glu Arg Ile Gly Pro Tyr Met Lys Val Ala Pro Pro Val
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Arg Ser Ala Glu Met Asn Val Arg Leu Trp Glu Gln Leu Glu Asn Gly
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Val Ile Asp Thr Leu Gly Ser Asp His Gly Gly His Pro Val Glu Asp
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Lys Glu Pro Gly Trp Lys Asp Val Trp Lys Ala Gly Asn Gly Ala Leu
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Ser Asp Ala Asp Leu Leu Ile Leu Asp Leu Asp Ile Asp Thr Lys Val
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Asp Ala Ser Gln Phe Arg Ser Leu His Lys Tyr Ser Pro Phe Asp Gly
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Met Pr
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08473

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/55 C12N15/61 C12P13/00 C12P13/22 C12P41/00
C12N1/21 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EPO-Internal, WPI Data, PAJ, STRAND, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WATABE K ET AL: "IDENTIFICATION AND SEQUENCING OF A GENE ENCODING A HYDANTOIN RACEMASE FROM THE NATIVE PLASMID OF PSEUDOMONAS-SP STRAIN NS671" JOURNAL OF BACTERIOLOGY, vol. 174, no. 11, 1992, pages 3461-3466, XP000944037 ISSN: 0021-9193 page 3461, left-hand column, paragraph 2 -page 3463, right-hand column, paragraph 2; figures 3,4 page 3465, right-hand column page 3466, left-hand column, paragraphs 3-5	1-3,5,6, 11
Y	page 3465, right-hand column, paragraph 1 -page 3466, left-hand column, paragraph 1	1-3,5,6, 11,13
Y	page 3462, left-hand column, paragraph 5 -page 3462, right-hand column, paragraph 1	9
Y	page 3462	4

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2000	16. 01. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Steffen, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GRIFANTINI RENATA ET AL: "Efficient conversion of 5-substituted hydantoins to D-alpha-amino acids using recombinant Escherichia coli strains." MICROBIOLOGY (READING), vol. 144, no. 4, April 1998 (1998-04), pages 947-954, XP002154848 ISSN: 1350-0872 page 949; figure 1 page 950, right-hand column, paragraph 2 -page 952, right-hand column, paragraph 1; table 4 page 953, left-hand column	1-3,5,6, 11,13
X	SIEMANN MARTIN ET AL: "Characterization of serological properties of polyclonal antibodies produced against enzymes involved in the L-selective cleavage of hydantoin derivatives." BIOTECHNOLOGY LETTERS, vol. 15, no. 1, 1993, pages 1-6, XP000944249 ISSN: 0141-5492 page 1, paragraph 1 -page 2, paragraph 4 page 5; table 1	14,16
Y	page 2; table 1	4
X	WILMS BURKHARD ET AL: "Cloning, nucleotide sequence and expression of a new L-N-carbamoylase gene from Arthrobacter aurescens DSM 3747 in E. coli." JOURNAL OF BIOTECHNOLOGY, vol. 68, no. 2-3, 19 February 1999 (1999-02-19), pages 101-113, XP004164275 ISSN: 0168-1656 page 102, right-hand column, paragraph 4 -page 103, left-hand column, paragraph 1 page 109, left-hand column	14,16
Y	page 103 -page 104, left-hand column	9
X	BLATTNER FREDERICK R ET AL: "The complete genome sequence of Escherichia coli K-12." SCIENCE (WASHINGTON D C), vol. 277, no. 5331, 1997, pages 1453-1462, XP002069950 ISSN: 0036-8075 page 1454, right-hand column, paragraph 3	15

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6

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE GENBANK 'Online! NCBI; Acc No: J01566, 8 February 1996 (1996-02-08) LEBOWITZ, J.: "Plasmid ColE1, complete genome" retrieved from NCBI, accession no. http://www.ncbi.nlm.nih.gov:80/ Database accession no. http://www.ncbi.nlm.nih.gov:80/Genbank/index.html XP002154850 the whole document	15
P,X	MAY OLIVER ET AL: "Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine." NATURE BIOTECHNOLOGY, vol. 18, no. 3, March 2000 (2000-03), pages 317-320, XP002154849 ISSN: 1087-0156 page 318, right-hand column, paragraph 2 -page 319, left-hand column, paragraph 1 page 319, right-hand column, paragraph 3 -page 320, left-hand column, paragraph 1	1-16
P,X	WIESE ANJA ET AL: "Hydantoin racemase from Arthrobacter aurescens DSM 3747: Heterologous expression, purification and characterization." JOURNAL OF BIOTECHNOLOGY, vol. 80, no. 3, 2000, pages 217-230, XP000943983 ISSN: 0168-1656 page 220, right-hand column, paragraph 2 -page 222, left-hand column, paragraph 1; figures 2,4	1-16
A	WATABE K ET AL: "CLONING AND SEQUENCE OF THE GENES INVOLVED IN THE CONVERSION OF 5-SUBSTITUTED HYDANTOINS TO THE CORRESPONDING L AMINO ACIDS FROM THE NATIVE PLASMID OF PSEUDOMONAS-SP STRAIN NS671" JOURNAL OF BACTERIOLOGY, vol. 174, no. 3, 1992, pages 962-969, XP000944036 ISSN: 0021-9193 the whole document	

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/08473

Box I Observations wher certain claims wer found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for th following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6,8,9,13 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6,8,9,13 (partially)

Present claims 6, 8, 9 and 13 relate to an extremely large number of possible methods. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely when the whole cell catalyst as referred to in claims 6, 8, 9 and 13 is restricted to a whole cell catalyst according to claim 1.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.